VIP Protein Folding

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Strategic Use of Non-native Diselenide Bridges to Steer Oxidative Protein Folding**

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Oxidation of cysteine thiols to disulfides is a widespread posttranslational modification of exported proteins, including many of pharmaceutical interest. For disulfide-rich proteins, many different disulfide-bonded species are possible, and the pathway from a reduced unfolded polypeptide chain to the fully oxidized, native conformation may entail multiple oxidation, reduction, and rearrangement steps.[1] Not surprisingly, formation of scrambled disulfide isomers or accumulation of kinetically and conformationally trapped intermediates often limits the rates and yields of oxidative protein folding. Biotechnological production of such proteins would consequently benefit from strategies that improve folding efficiency.

We recently showed that redox buffers containing selenoglutathione (GSeSeG), which is a diselenide analogue of glutathione (GSSG), promote the oxidative folding of a wide range of proteins.[2] Diselenides are thermodynamically more stable than disulfides[3] but react more rapidly with thiols owing to the higher polarizability of selenium compared to sulfur.[4] The greater acidity of selenols versus thiols ($\Delta pK_a \approx 3$)[5] further enhances their reactivity. For example, deprotonated selenolate anions are excellent nucleophiles that are capable of catalyzing disulfide bond shuffling.[4] As a consequence, diselenide-containing redox buffers typically afford higher rates and yields of native protein than conventional glutathione redox buffers over a broader pH range and at substantially lower concentrations.[6] Because selenols are rapidly oxidized by molecular oxygen, GSeSeG can even function as a true catalyst for aerobic folding reactions.[7]

The utility of GSeSeG is particularly striking for proteins, such as bovine pancreatic trypsin inhibitor (BPTI), whose folding mechanisms are dominated by kinetically trapped species.[2] Diselenide-containing redox buffers rescue such intermediates, much like the natural enzyme protein disulfide isomerase (PDI),[8] by accelerating rate-limiting disulfide bond rearrangements.[9] While intermolecular reactions of diselenides and reduced protein can be quite efficient, intramolecular reactions would be expected to provide even greater control over oxidative folding pathways. In fact, diselenides have been widely used as isosteric replacements of native disulfides in cysteine-rich proteins, such as endothelins[10] and conotoxins,[11] to increase folding rates and yields while maintaining biological activity. Because diselenides have significantly lower reduction potentials than disulfides,[3] their formation is favored thermodynamically, providing a powerful intramolecular constraint that ensures regioselective formation of correct cross-links. Moreover, the diselenides increase the stability of these molecules to reduction and disulfide scrambling.[11a,d] In contrast, *non-native* diselenide cross-links are useful for generating thermodynamically stable models of kinetically unstable folding intermediates. For example, non-productive conformations of the 18-amino acid peptide apamin have been successfully trapped by this approach.[12] For proteins more stable than apamin, however, incorrectly cross-linked intermediates need not necessarily be kinetic dead ends, provided sufficient conformational folding energy is available to override the thermodynamic benefit associated with diselenide formation. We hypothesized that it might even be possible to exploit non-native diselenides in such cases to bias early folding events and strategically direct folding toward a specific productive route.

BPTI, one of the first proteins for which a detailed folding pathway was deduced,[13,14] is 58 amino acids long and stabilized by three disulfide bonds, which are designated by the positions of the paired cysteines, i.e. [5–55; 14–38; 30–51] (Figure 1a). The reduced protein folds in the presence of glutathione via a bifurcated pathway involving a small number of discrete intermediates that contain one and two native disulfide bonds (Figure 1b).[14] Roughly half of the reduced BPTI molecules reach the native state via an intermediate (N′) that lacks the 5–55 disulfide; the other half are trapped as a stable, native-like species (N*) that lacks the 30–51 disulfide. Formation of the fully oxidized native state requires partial unfolding and subsequent rate-limiting disulfide bond rearrangements of these intermediates. At neutral pH, N* is stable for weeks, greatly limiting folding efficiency.[13,14]

In the early stages of BPTI folding, a broad spectrum of one-disulfide intermediates is initially formed, but the population quickly becomes dominated by species containing native 5–55 and 30–51 disulfides.[14,15] We speculated that replacing both Cys5 and Cys14 with selenocysteine (usually abbreviated as Sec or U) would perturb this normal steady-state distribution owing to preferential formation of the non-native 5–14 cross-link. Biasing the system in this way might further influence the set of two-disulfide intermediates that arise and thus open alternative folding channels to the fully oxidized protein (Figure 1b). For example, the 5–14 diselenide would be expected to stabilize the non-native [5–14; 30–51] intermediate, which has been detected at low levels in

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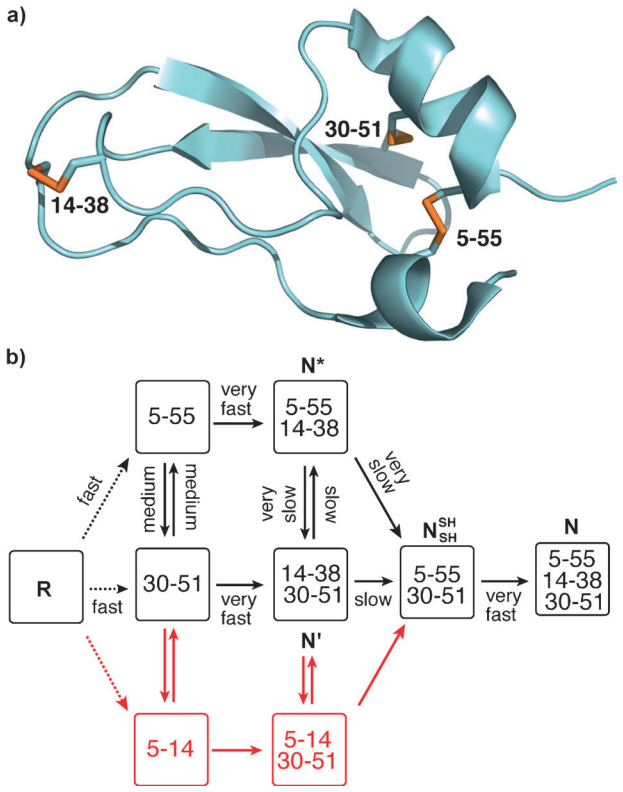

Figure 1. Bovine pancreatic trypsin inhibitor (BPTI). a) Structure of the folded protein (PDB entry: 1BPI) showing the three native disulfide bonds, 5–55, 14–38, and 30–51 (orange). b) Kinetically favored BPTI folding pathway (black).[14] R and N are reduced and native BPTI, respectively; intermediates that normally accumulate during folding are designated by the disulfide bonds they contain. Initial oxidation of the reduced protein affords a broad distribution of single disulfide intermediates (dotted lines) that rearrange to give [5–55] and [30–51]. Qualitative estimates of the relative rates of individual steps are indicated above or below the arrows. Introduction of a non-native 5–14 diselenide constraint into the protein could provide alternative pathways to N; one possibility is shown in red.

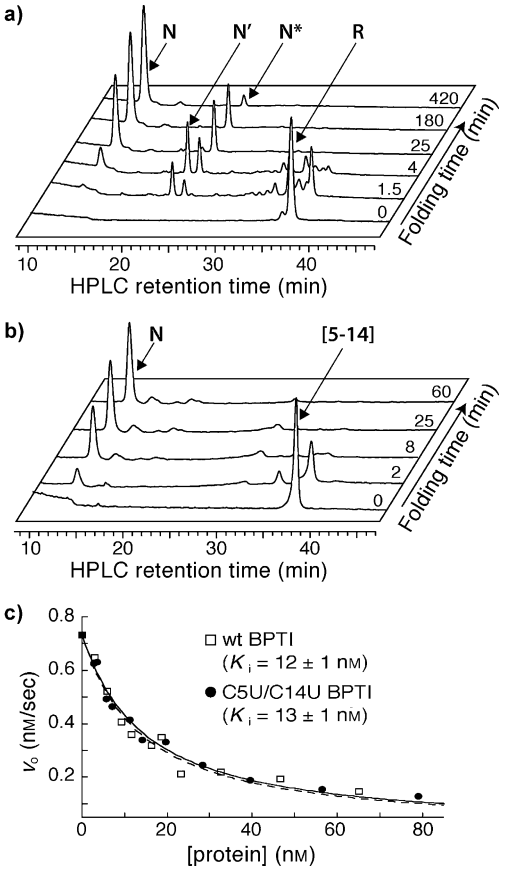

Figure 2. Anaerobic folding of BPTI variants at pH 8.7. a),b) Representative HPLC chromatograms for acid-quenched aliquots from folding reactions with wt BPTI and C5U/C14U BPTI, respectively. [protein] = 30 μM; [GSSG] = 150 μM. c) Inhibition of bovine α-chymotrypsin by native wt BPTI (□) and by C5U/C14U BPTI (●). The data were fitted as described in the Supporting Information to give the apparent K_i values indicated.

kinetic folding experiments with wild-type (wt) BPTI but is less stable than either N′ or N*.[13a,16] This species is interesting because a single thiol–disulfide-type exchange step would convert it into [5–55; 30–51], also known as N^{SH}_{SH}, which is rapidly oxidized to N (Figure 1b). To investigate how increasing the amount of a non-native cross-linked species might influence BPTI folding, we chemically synthesized the C5U/C14U variant by a three-fragment native chemical ligation (NCL) strategy[17] (Supporting Information, Figures S1–S3). The selenoprotein was obtained in milligram amounts, purified by HPLC, and characterized by mass spectrometry. Because of the facile oxidation of selenols in air, the protein was isolated in homogeneous form with a single diselenide cross-link and four reduced thiols.

Folding experiments with C5U/C14U BPTI were carried out anaerobically using 30 μM protein and 150 μM GSSG as an oxidant at pH 8.7 and 25 °C, as previously described.[14] Unlike non-native diselenides in apamin,[12] the 5–14 diselenide in BPTI does not impede formation of the native state. Rather, the selenoprotein folds roughly twice as fast as wt BPTI (Figure 2a versus b). Strikingly, though, neither N′ nor N*, the kinetically trapped intermediates that define the normal BPTI folding pathway, are observed. Instead, several broad peaks appear between 30 and 35 min in HPLC traces of the acid-quenched folding mixture (Figure 2b). Although unambiguous identification of the individual intermediates formed was not possible, this is the region of the chromatogram where diverse one- and two-disulfide intermediates normally appear, including N^{SH}_{SH} and the non-native species [5–14; 30–51] and [5–38; 30–51].[14] The broadness of these peaks contrasts with the relatively sharp peaks for the starting material and final product, suggesting a highly heterogeneous mixture of species that is converted directly into the native state without populating N′ or N*. Alternatively, N′ and N* may still form transiently during folding of C5U/C14U BPTI but are no longer kinetically trapped because the selenium substitution speeds up their conversion into the native state. Additional experiments will be required to distinguish between these two possibilities. Importantly, though, the folded and fully oxidized C5U/C14U BPTI exhibits CD spectra (Supporting Information, Figure S4) and inhibition profiles (Figure 2c) that are indistinguishable from wt BPTI,

even though it contains two selenosulfides in place of the native [5–55] and [14–38] disulfides.

Qualitatively similar behavior is observed at neutral pH where the oxidation of BPTI by GSSG is considerably slower (Figure 3). Under these conditions, C5U/C14U BPTI reaches the native state appreciably faster than wt BPTI (Figure 3a), again without detectable formation of either N′ or N* (Supporting Information, Figure S5). After 30 h, the yield of fully oxidized protein is 72%, which compares to 65% for wt BPTI after 5 days (Figure 3b versus c). The selenoprotein also folds efficiently in air in the absence of GSSG (Figure 3a,d), giving 70–80% native protein after 24 h. In contrast, wt BPTI folds extremely slowly under these conditions. After 28 h, only 12% of the protein has attained the native state; the remainder accumulates as the N′ (14%) and N* (66%) intermediates (Figure 3e). Apparently, the C5U/C14U diselenide bond serves as an effective intramolecular catalyst of BPTI folding,[18] enhancing the rate of oxidation substantially by avoiding or eliminating the N′ and N* kinetic traps.

The most dramatic effects, though, are seen when the selenoprotein is oxidized with GSeSeG at pH 7.3 (Figure 3a). In the presence of this reagent, folding of C5U/C14U BPTI affords N in about 85% yield within 6 h (Figure 3f). Although N is formed significantly faster than in the reactions with GSSG or molecular oxygen, the mechanism of the reaction appears to be the same as with the other oxidants, as neither N′ nor N* is detected (Supporting Information, Figure S5). GSeSeG has been shown to promote the folding of wt BPTI by accelerating the conversion of N* to N in the later stages of the folding reaction,[9] but in contrast to the selenoprotein, little kinetic advantage is evident at early time points (Figure 3a). As a consequence, less than 20% of wt BPTI has reached the native state after 5 h (Figure 3g). Transient formation of GSeH during the initial oxidation step may account for the remarkable synergy between the selenoprotein and the external diselenide oxidant, as selenol/diselenide exchange reactions are many orders of magnitude faster than analogous thiol/disulfide exchange reactions.[4,19] Nevertheless, neither the folding conditions nor the concentration or composition of the redox buffer have been optimized, so further increases in folding efficiency and yield of native protein might be achievable by systematic variation of these parameters.[21]

The importance of non-native states in BPTI folding has been controversially discussed.[14,20] Our results indicate that deliberate population of such species by a non-native diselenide constraint can profoundly influence the folding pathway, altering the balance of productive and non-productive routes and substantially increasing folding efficiency under all conditions tested. This strategy thus provides a potentially general means of rationally engineering folding mechanisms to favor specific productive routes. Although folding has also been enhanced by optimizing redox buffers[21] and by destabilizing unproductive intermediates through mutagenesis,[22] the use of a diselenide for this purpose is attractive because of the minimal structural perturbation involved and the possibility of catalyzing both the oxidation and isomerization steps leading to the fully oxidized native state. Systematic analysis of all possible natural and non-natural diselenides should allow rapid optimization of folding conditions. Because selenocysteine is a natural proteinogenic amino acid,[23] there is also potential for producing diselenide-containing variants recombinantly.[24] The synergy resulting from combining an internal protein diselenide with an external diselenide oxidant appears to be particularly powerful method for shortening reaction times and may thus prove useful for diverse folding applications.

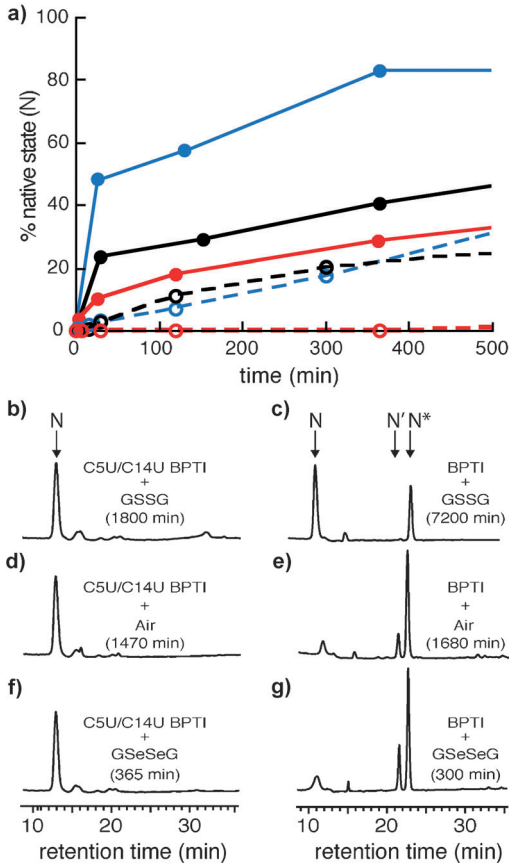

Figure 3. Oxidative folding at pH 7.3. a) Kinetic traces of the initial stages of the folding reactions of wt BPTI (-----) and C5U/C14U BPTI (——) in the presence of 150 μM GSSG (black), air (red), or 150 μM GSeSeG (blue). [protein] = 30 μM. b)–g) Representative HPLC chromatograms for acid-quenched aliquots of the folding reactions of C5U/C14U BPTI and wt BPTI with GSSG, air, and GSeSeG.

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Communications

Protein Folding

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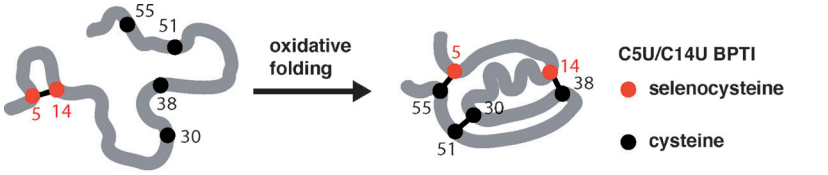

Targeted insertion of a non-native diselenide cross-link into a cysteine-rich protein can be exploited to direct the early stages of oxidative folding so as to avoid accumulation of unproductive intermediates that limit folding efficiency. This simple strategy could facilitate the production of many difficult-to-fold peptides and proteins.